Biodiverse Responses of O-methyltransferase Genes to Salt Stress and Fiber Development of Gossypium Species

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Abstract

O-methyltransferases (OMTs) are an important group of enzymes that catalyze the transfer of a methyl group from S-adenosyl-L-methionine to their acceptor substrates. OMTs are divided into several groups according to their structural features. In *Gossypium* species, they are involved in phenolics and flavonoid pathways. Phenolics defend the cellulose fiber from dreadful external conditions of biotic and abiotic stresses, promoting strength and growth of plant cell wall. In this study, an *OMT* gene family, containing a total of 192 members, has been identified and characterized in three main *Gossypium* species, *G. hirsutum*, *G. arboreum* and *G. raimondii*. Cis-regulatory elements analysis suggested important roles of *OMT* genes in growth, development, and defense against stresses. Transcriptome data of different fiber developmental stages in Chromosome Substitution Segment Lines (CSSLs), Recombination Inbred Lines (RILs) with excellent fiber quality, and standard genetic cotton cultivar TM-1 demonstrate that up-regulation of *OMT* genes at different fiber developmental stages, and abiotic stress treatments have some significant correlations with fiber quality formation, and with salt stress response. Quantitative RT-PCR results revealed that *GhOMT43* and *GhOMT27* genes had a specific expression in response to salt stress while *GhOMT16*, *GhOMT55*, and *GhOMT33* in fiber elongation and secondary cell wall stages. Our results indicated that these genes might contribute to salt tolerance or fiber quality traits respectively in *Gossypium*.

1. Introduction

Cotton (*Gossypium* Species) has the importance for natural fiber all over the globe. The primary goals of upland cotton (*G. hirsutum*) perspectives have been always to achieve better quality with higher yield (Lee et al. 2007). Mostly *G. hirsutum* bears staple fibers 25–40 mm in length and 15 µm in thickness at their full maturity. Fiber cells must undergo four distinct but partially overlapped developmental stages, including initiation, elongation, secondary cell wall deposition, and maturation. The secondary cell wall of fiber, which is mainly composed of cellulose, is important especially for fiber quality perspectives. However, some studies have shown that secondary cell wall of fibers of flax (*Linum usitatissimum* L.), ramie (*Boehmeria nivea* L.), and Spanish broom (*Spartium junceum* L.) also contain phenolics along with cellulose. Their fibers are known for their physical properties such as length and strength and have been used for textile purposes. A thicker secondary cell wall was estimated to contain no less than 70% cellulose content while the cotton fiber contains almost 90% cellulose (Angelini et al. 2000; Day et al. 2005). Lignin is another important component in cell wall (Zhang et al. 2020a). It provides strength to plant cell wall and response to biotic and abiotic stresses in vascular plants (Liu 2012). The presence of lignin, which is reported at lower level in secondary cell wall of cotton fibers (Fan et al. 2009), negatively regulates fiber elongation and secondary cell wall synthesis in cotton. Studies demonstrated that the cotton plants that accumulate less lignin and lignin-like phenolics in mature fibers tend to have longer and stronger fibers (Gao et al. 2019). From an active perspective, lignin and phenolics defend the cellulose fiber against dreadful conditions and increase the ability of response to biotic and abiotic stresses, and thus influence the growth and strength of plant cell walls (Boerjan et al. 2003). Previous studies in herbaceous plants demonstrated the involvement of O-methyltransferases (*OMTs*) in lignin biosynthesis (Zhong 1998). The involvement of *OMTs* mediate normal plant growth in the presence of lignin (Ye and Varner 1995). The initial *OMT* cDNA was described in 1991 (Bugos et al. 1991), then a series of *OMT* cDNAs have been cloned from diverse plants species, including *Zea mays*, *Arabidopsis thaliana*, *Iris hollandica*, and *Nicotiana tabacum* (Vincent et al. 2005).

According to substrate classification, plant methyltransferases have three major categories, I. O-methyltransferases (*OMTs*), II. N-methyltransferases (*NMTs*), and III. C-methyltransferases (*CMTs*). The category I OMTs are further classified into five sub-categories. Sub-category I-a comprises caffeoyl coenzyme A 3-O-methyltransferase (*CCoAOMT*) and caffeic acid 3-O-methyltransferases (*COMTs*), which are involved in methylation in phenylpropanoids. Sub-categories I-b, I-c, and I-d act in methylation of hydroxyl in flavonoid, alkaloids, and myoinositol, respectively. The fifth sub-category I-e takes part in methylation of carboxyl of diverse acids. The results of a study (Zubieta et al. 2002) discovered the crystal structure of *OMTs* from *Medicago sativa*. In the light of the explanations, the *OMT* gene that was cloned and characterized from a medicinal plant *Ligusticum chuanxiong* and contained higher ferulic acid was named as *LcCOMT*. The *LcCOMT* gene was differentially expressed under cold stress treatments, which suggested that it can assemble the ferulic acid under chilling stress. BLAST analysis identified 23.9–40.2% similarity of *LcCOMP* with the *OMTs* of alkaloid, flavonoid, isoflavonoid, and phenylpropanoids (Li et al. 2015).

In the whole life cycle of cotton plant, it undergoes various environmental conditions from the early spring in April when it is sowed to mid-summer when it grows rapidly in vegetation and reproduction and to late autumn when it gets mature and is harvested. During the whole growth procedure, the cotton plant maintains an exquisite molecular controls and regulations. But little is known what roles the *OMT* family genes have played in cotton plant especially in early or late growth stage when season transition occurs, or in various stress conditions. Therefore, in this study, we identified the *OMT* family genes in the genome-wide scale and made detailed bioinformatics

analysis of gene structure, chromosomal distribution, selection pressure during their evolution, sub-cellular localization, cis-regulatory elements etc, together with their expression profiling in different developmental stages and in responses to various stresses. Their expression profiling in developing fiber cells was verified using RNA sequencing data from RILs, CSSLs, and TM-1 at different fiber development stages. This study could open the way to comprehend the functions of *OMTs* in fiber quality advancement and in cotton plant responses to abiotic stresses, and thus could assume a noteworthy part for further investigation in the molecular mechanism of fiber improvement and stress tolerance.

2 Materials And Methods

2.1 Databases

Genome files of three *Gossypium* species including *G. arboreum* (CRI), *G. raimondii* (JGI), and *G. hirsutum* (NAU) were downloaded from cotton functional genomic database (http://www.cottonfgd.org). The protein sequences of *A. thaliana* and *T. cacao* were retrieved from the Phytozome database https://phytozome.jgi.doe.gov/pz/portal.html.

2.2 Identification of *OMT* protein family members, sequences alignment, and phylogenetic tree construction

The hidden Markov model (HMM) (PF00891 and PF01596) was downloaded from Pfam (https://pfam.xfam.org/). The HMMER 3.0 software was used to acquire the OMT genes of Pfam (PF00891 and PF01596) with default parameters. Then the evaluated genes were confirmed by using Pfam (https://pfam.xfam.org/) and SMART (Simple Modular Architecture Research Tool) (Khan et al. 2018). After confirmation of evaluated results, Manual check for the presence of methyltransferase domains performed. Members with the absence of required domains were manually removed, while some potential OMT genes were retrieved according to some other features including chromosomal positions, protein length (aa), and molecular weight (kDa) by using cotton functional genomic database (http://www.cottonfgd.org/). The full length amino acid sequences of *G. hirsutum*, *G. arboreum*, *G. raimondii*, *A. thaliana*, and *T. cacao* encoded by OMT genes were aligned with clustalx2 software (http://www.clustal.org/)(Arai et al. 2019) with default parameters for the neighbor-joining phylogenetic tree as 1000 bootstraps. Subsequently, two neighbor-joining phylogenetic trees were generated by using Mega 7 (Khan et al. 2018). The topology of both phylogenetic trees was confirmed to understand the phylogenic relationship within the five plant species.

Nomenclature of these members was based on their chromosomal locations and numbers in each *Gossypium* species.

2.3 Chromosomal mapping and collinearity analysis

Gene IDs were used for blast within cotton genome files to estimate the positions of OMT genes. The physical positions of OMT genes in three cotton species were visualized by using TBtools software (Wu et al. 2019). Circle gene viewer model of TBtools software was used to visualize collinearity between homologous sequences.

2.4 Gene structure and conserved motifs

The structure of the *OMT* genes was analyzed using the online server of Gene Structure Display (GSDS 2.0, http://gsds.cbi.pku.edu.cn) (Fan et al. 2019). The conserved motifs were predicted online in MEME web based motif prediction tool version 5.0.5 (http://meme-suite.org/) by providing protein sequences of OMT genes (Khan et al. 2018).

2.5 Selection pressure, cis-regulatory elements, sub-cellular localization and gene enrichment analysis

The CDS of homologous gene pairs of *G. hirsutum* (NAU), *G. arboreum* (CRI), and *G. raimondii* (JGI) were assigned to TBtools software to estimate the ka/ks ratio to predict selection pressure between the genes of each pair in genomes and sub-genomes (Wu et al. 2019). The 2000 bp upstream sequences of *OMT* genes were submitted to PlantCARE database (Fan et al. 2019) to obtain the cis-regulatory elements. To predict the Sub-cellular localization of genes was predicted using online bioinformatics tool CELLO v.2.5 with their protein sequences (Yu et al. 2004). Original IDs of OMT family members were submitted to search for functional annotations on Cotton functional genomic database. Gene ontology and KEGG pathways were performed to evaluate the *OMT* functional annotations with TBtools blast against enrichment databases.

2.6 Expression profiling of OMTs

The different sets of RNA sequencing data including TM-1, a genetic standard line of *G. hirsutum* (Nanjing Agricultural University, Nanjing, Jiangsu, China) (PRJNA248163) (Hu et al. 2019; Zhang et al. 2015), 69307 and 69362 (selected lines from a RIL population sGK9708 × 0-153, Institute of Cotton Research, Anyang, Henan, China) (PRJNA542946) (Zhang et al. 2020b), MBI7747, MBI7561, and MBI7285 (selected lines from CSSL population CCRI45 × Hai1, SRP084203) (Lu et al. 2017; Shi et al. 2015), and MBI9915 and MBI9749 (selected lines from CSSL population CCRI36 × Hai1, SRX2843778) (Institute of Cotton Research, Anyang, Henan, China) (Li et al. 2017; Shi et al. 2015) were included in this study to observe the expression pattern of OMT family genes at different growth stages, under abiotic stress treatment stages, ovule development, and in different fiber development stages of cotton. Briefly, 69307, 0-153, MBI7747, MBI7561, MBI9915, MBI9749, and Hai1 have high fiber quality traits, while 693062, MBI7285, sGK9708, CCRI36, and CCRI45 have low fiber quality traits. Detailed information of these referenced materials is presented in S1 Table.

Transcriptome data of *G. arboreum* (PRJNA179447) (Zhang et al. 2015), and *G. raimondii* (PRJNA79005) (Wang et al. 2012) were also included to compare the comparative expression of these OMT genes.

2.7 Plant material, RNA isolation, cDNA synthesis, and qRT-PCR

Cultivars sGK9708 and 0-153 (S1 Table) were planted in April 2018 in the experimental fields of the Institute of Cotton Research, Chinese Academy of Agricultural Sciences, Anyang, Henan. Flowers were tagged on the day of anthesis for fiber sampling in July 2018. Bolls of tagged flowers were sampled in the morning between 9:00 and 10:00 AM at 10, 15, 20, and 25 days' post anthesis (DPA). The fibers were dissected from the developing seeds right after boll picking and immediately stored at -80 ^{o}C for RNA extraction.

To examine the expression profiling of *OMT* genes under salt stress, seeds of sGK9708 cultivar were germinated in wet filter papers for 72 hours and then were transferred to hydroponic conditions. The seedlings were treated with 200 mM NaCl at three leaves stage. The true leaves, stems, and roots were sampled at 0hr, 2hrs, and 6hrs of the treatment. The 0 h of treatment was considered as control sample to compare the expression profiling with treated samples.

Total RNA isolation was performed with the RNAprep Pure Plant Kit by (Tiangen, Beijing, China). To eliminate the genomic DNA contamination, the RNA samples were treated with DNase1. RNA concentration and integrity was observed on Nano Drop 2000 spectrophotometer (Thermo scientific, USA) and 1% agarose gel electrophoresis. cDNAs of the RNA samples that the A260/280 ratio reached 2.00 were synthesized using PrimeScript® RT Reagent Kit (Perfect Real Time, Takara Biotechnology Co., Ltd., Dalian, China). qRT-PCR was performed with ABI 7500 fast Real-Time PCR system (Applied Biosystems, USA), with *Gh-Histone3* gene was used as reference to normalize the relative expression level. Primers pairs of five *OMT* genes were designed by using Oligo 7 (S2 Table). $2^{-\Delta\Delta}$Ct method was used to calculate the gene expressions (Fan et al. 2019).

3 Results

3.1 Characterization of putative OMT genes

The genome wide analyses revealed that the *OMT* family genes have varying sizes and diverse annotations in *Gossypium* species (S3 Table. Sheet A). There various features and characterizations of *OMTs* in *Gossypium* might result in differentiation of functions of this gene family. A total of 192 *OMT* members were identified in three *Gossypium* species, including 82 in *G. hirsutum*, 55 in *G. arboreum*, and 55 in *G. raimondii* (S3 Table. Sheet A). For phylogenetic analysis (Ma et al. 2016a), 33 *OMT* members in *A. thaliana*, and 26 members in *T. cacao* species were also identified (https://phytozome.jgi.doe.gov/pz/portal.html) (S3 Table. Sheet B). Retrieving information of *OMT* genes in *G. hirsutum* revealed that *GhOMT81*, which was detected in scaffold, coded the smallest peptide of 62 amino acids (aa) with a molecular weight of 6.642 kDa. While *GhOMT40*, which was identified on chromosome D_t02, coded the largest peptide of 969 aa with a molecular weight of 108.296 kDa among all *OMT* members in three *Gossypium* species.

As domain wise characterizing *OMT* family genes in *Gossypium* species, the results revealed that almost 3/4 (*G. hirsutum*) to more than 4/5 (*G. arboretum and G. raimondii*) of the *OMT* family genes harbored methyltransf_2 domain, while only from 1/4 to less than 1/5 harbored methyltransf_3 domain. As comparison, a little less than 2/3 of the OMT family genes in *A. thaliana* harbored methyltransf_2 domain and in *T. Cacao*, this number was almost 2/2.

3.2 Chromosomal distribution, collinearity, duplication, and loss of OMT genes

We confirmed chromosomal locations of the *OMT* family genes in *Gossypium* as previously described (Zhang et al. 2019). A total of 161 *OMT* genes were positioned on their respective chromosomes, while seven of *G. raimondii*, one of *G. arboreum*, and 23 of *G. hirsutum* were positioned in scaffolds (S4 Figure). In *G. raimondii* (D genome), chr11 was mapped with 13 genes followed by chr08 with nine genes. The minimum number of genes in a chromosome was one in chr2, chr6, and chr10 respectively. There was no *OMT* family members identified in chr01 and chr07 (S4 Figure.a). In *G. arboreum* (A-genome) (S4 Figure.b), 54 *OMT* genes were mapped in all chromosomes except chr1. Chr10 harbored 13 *OMT* genes which were the highest per chromosome, followed by chr12 and chr04 with 10 and 9 genes respectively. The minimum number of genes located in a chromosome was one in chr02 and chr11 respectively. In *G. hirsutum* (A_tD_t genome) (S4 Figure.c), unexpectedly, there were no *OMT* genes in A_t02, A_t05, A_t07, D_t03, D_t09, and D_t11 chromosomes. The distribution of genes in D_t sub-genome (33 genes) was higher than in A_t sub-genome (26 genes). The maximum number of genes in a chromosome was seven in D_t04 and A_t12, followed by four in D_t10 and A_t10 chromosomes, respectively. D_t01, D_t05, A_t01, A_t06, and A_t11 only had one *OMT* gene, and D_t06, D_t07, A_t03, A_t08, A_t09, A_t13 two *OMT* genes and D_t02, D_t08, and D_t13 three *OMT* genes respectively (S4 Figure.c). A collinearity analysis of the *OMT* family genes in *Gossypium* species chromosomes was shown in Fig. 1. The results demonstrated a pair wise collinearity of *OMT* genes between the chromosomes on which *OMT* family genes were mapped. Noticeably, a number of available genes in A_t and D_t scaffolds were collinear with their homologues in A and D genomes suggesting the collinearity of the DNA fragments between the scaffolds and chromosome where these *OMT* genes locate (Fig. 1). Taken the *OMT* gene numbers identified in each A/D genome or A_t/D_t sub-genome, collinearity analysis also revealed that there were totally 21 and 19 *OMT* genes exclusively detected in A and D genomes respectively. Their homologous counterparts in A_tD_t genomes of *G. hirsutum* are lost. There are also a few OMT genes that are exclusively detected in A_tD_t genome of *G. hirsutum* without homologous counterparts in A and D genomes (S4 Figure).

According to studies there are five types of duplications including singleton, dispersed, proximal, tandem, and segmental or whole-genome duplication (Qiao et al. 2019). A total of 73 members of the *OMT* genes of three *Gossypium* species were identified to have dispersed duplications, fifty-one genes (including 15 in A genome, 12 in D genome, and 24 in A_tD_t genome) to have segmental duplications, while thirty-four to have singleton duplications (S5 Table Sheet A).

3.3 Analysis of selection pressure

The ratio of the number of non-synonymous substitutions per non-synonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks) represents selection pressure of the gene (Ma et al. 2016b). $Ka/Ks < 1$ demonstrates high purifying selection pressure, while $Ka/Ks > 1$ shows positive selection pressure. Analysis of Ka/Ks ratio of homologous *OMTs* in three *Gossypium* species revealed that they are under purifying selection pressure. The Ka/Ks ratio of homologous *OMTs* in *G. raimondii* and *G. arboreum* ranged from 0.09 to 0.8, in *G. raimondii* and *G. hirsutum* ranged 0 to 0.7, and in A_t and D_t of *G. hirsutum* ranged 0.4 to 0.7 (S5 Table Sheet B).

3.4 Phylogenetic Analyses, Sequences alignment, conserved motifs and gene structure

The phylogenetic analysis included all of the 251 *OMT* genes identified in this study, including 192 genes from three *Gossypium* species (Fig. 2.a), 33 from *A. thaliana*, and 26 from *T. cacao* species (Fig. 2.b). The evolutionary relationship of *OMT* genes in three *Gossypium* species was closer and more similar with each other (Fig. 2.a) than with *A. thaliana* and *T. cacao* (Fig. 2.b). According to the topology of constructed tree, the *OMT* gene family is divided into five clades (I, II, III, IV, and V) in *Gossypium*, *A. thaliana*, and *T. cacao* species. Previous study has also identified five clades of *OMT* genes in *Catalpa bungei* (Lu et al. 2019). The results showed that each clade of *OMT* genes were symmetrically distributed within *Gossypium* species (Fig. 2.a), while in *A. thaliana* and *T. cacao*, *OMT* genes were identified in cluster forms (Fig. 2.b). The results demonstrated that these *Gossypium OMT* members might be evolutionary close within respective species and their identified clades.

To examine the conserved motifs of each clade, the analysis of representative motif logo and conserved motifs prediction were conducted (S6 Figure). The results revealed that motif 1, enriched with leucine, valine, and glycine, motif 2, enriched with leucine and valine, and motif3, motif 4, motif 5, and motif 6 were common in clades I, II, III, IV and clade V. While motif 7 was found missing in some members of clade V, which was then replaced with motif 8 at same positions (S6 Figure). The enriched amino acid residues of conserved motif1 (L/VDVGGG/TG) was previously identified in S-adenosyl-l-methionine (SAM)-dependant *OMTs* that shared 95% similarity with *G. hirsutum OMT* which further evidenced the biological function of identified genes in response to diverse abiotic stresses (Kim et al. 2013).

Methyltransferases serve different functions although they may have high sequence similarity. Investigation of gene structure has uncovered the different number of exons and introns of *OMT* genes. Exon and intron number of *OMT* genes varied from the least one exon and no intron to the most 7 to 9 exons and 6 to 8 introns (S7 Table). Two members in *G. hirsutum* including *GhOMT67* and *GhOMT30* contain nine exons as the highest (S7 Table). Same as, two members including *GaOMT47* in *G. arboreum*, and *GrOMT21* in *G. raimondii* contain 9 exons (S7 Table). Gene structure analysis revealed that the *OMT* genes with higher number of exons had a shorter exons and introns, and vise versa. These results demonstrated that *OMT* members possess different structural patterns in accordance with their features.

3.5 Identification of cis-regulatory elements in *OMT* family

The promoter regions of the *OMT* family contain precisely a large number of cis-regulatory elements. The analysis of cis-regulatory elements revealed the enrichment of MYB cis-regulatory elements, which was detected more than 350 times in *OMT* genes (S8 Figure). The MYC was another important element that was found 183 times in enlisted *OMT* genes. Box 4 (part of a conserved DNA module involved in light responsiveness) was found 152 times in 43/82 genes in *G. hirsutum*. ABRE elements was detected 119 times in 29/82 OMT genes in *G. hirsutum*. The ERE element was detected 113 times in 37/82and G-Box 97 times in 37/82 *G. hirsutum OMT* genes. An auxin RR-core and cis-acting regulatory element involved in the MeJA-responsiveness (TGACG-motif) were also observed in *Gossypium OMT* genes where this element was identified 48 times in 25/82 genes. Some other important cis-regulatory elements including wun-motif 44 times in 26/82, W-box 39 times in 31/82, GATA-motif 32 times in 27/82, O2-site 30 times in 22/82 *OMT* genes respectively, in *G. hirsutum* (S8 Figure). These cis-regulatory elements might function collectively in accordance with their specific roles and with specific conditions as well as growth and development stages (S8 Figure).

3.6 Sub-cellular localization prediction of *OMT* genes

Understanding and determining the sub-cellular localization of proteins is an important strategy to identify the function of protein at cellular level (Binder et al. 2014). This approach includes proteomic-based experiments and microscopic high throughputs (Andersen et al. 2002; Herold et al. 2009). Several sequence-based approaches have been developed to predict the sub-cellular localization by providing amino acid sequences including PSORT (Horton and Nakai 1997), Yloc (Briesemeister et al. 2010), BaCelLO (Pierleoni et al. 2006), LOCtree (Goldberg et al. 2012). According to Cello prediction, most of *OMT* genes were located in the cytoplasm (Table 1), while seven genes were predicted in periplasm, including, *GhOMT31*, *GhOMT33*, *GhOMT16*, *GhOMT55*, *GhOMT68*, *GhOMT69*, and *GhOMT71*. Five *OMTs* were predicted to be localized in both periplasm and cytoplasm, including *GhOMT32*, *GhOMT5*, *GhOMT50*, *GhOMT20*, and *GhOMT70*. Two genes *GhOMT67* and *GhOMT30* were predicted in the outer membrane. Only *GhOMT29* was predicted in inner membrane and cytoplasm (Table. 1). The function of the OMT genes might be related to their predicted localizations, though the experimental approach is still needed for further confirmation.

Table 1
Predicted Subcellular localization of *OMT* genes of *G. hirsutum*

Gene ID	Predicted Localization	Reliability*	Gene ID	Predicted Localization	Reliability*	Gene ID	Predicted Localization	Reliability*
GhOMT1	Cp	3.712	GhOMT29	IM/Cp	1.807/2.478	GhOMT57	Cp	2.352
GhOMT2	Cp	3.597	GhOMT30	OM	2.374	GhOMT58	Cp	4.675
GhOMT3	Cp	2.985	GhOMT31	Pp	4.34	GhOMT59	Cp	3.572
GhOMT4	Cp	3.897	GhOMT32	Pp/Cp	2.121/2.358	GhOMT60	Cp	2.968
GhOMT5	Pp/Cp	2.094/2.624	GhOMT33	Pp	4.154	GhOMT61	Cp	3.224
GhOMT6	Cp	4.545	GhOMT34	Cp	4.87	GhOMT62	Cp	4.112
GhOMT7	Cp	4.122	GhOMT35	Cp	4.493	GhOMT63	Cp	4.124
GhOMT8	Cp	2.707	GhOMT36	Cp	4.637	GhOMT64	Cp	4.385
GhOMT9	Cp	4.033	GhOMT37	Cp	3.636	GhOMT65	Cp	4.927
GhOMT10	Cp	4.822	GhOMT38	Cp	4.092	GhOMT66	Cp	2.781
GhOMT11	Cp	4.154	GhOMT39	Cp	4.228	GhOMT67	OM	2.226
GhOMT12	Cp	4.234	GhOMT40	Cp	3.13	GhOMT68	Pp	3.91
GhOMT13	Cp	4.275	GhOMT41	Cp	4.475	GhOMT69	Pp	2.49
GhOMT14	Cp	4.675	GhOMT42	Cp	4.619	GhOMT70	Pp/Cp	1.961/2.316
GhOMT15	Cp	4.743	GhOMT43	Cp	3.384	GhOMT71	Pp	4.276
GhOMT16	Pp	3.107	GhOMT44	Cp	4.717	GhOMT72	Cp	4.474
GhOMT17	Cp	3.838	GhOMT45	Cp	4.717	GhOMT73	Cp	4.312
GhOMT18	Cp	2.229	GhOMT46	Cp	4.735	GhOMT74	Cp	4.534
GhOMT19	Cp	1.577	GhOMT47	Cp	3.973	GhOMT75	Cp	4.641
GhOMT20	Pp/Cp	1.995/1.743	GhOMT48	Cp	3.924	GhOMT76	Cp	4.885
GhOMT21	Cp	3.577	GhOMT49	Cp	2.531	GhOMT77	Cp	3.973
GhOMT22	Cp	3.508	GhOMT50	Pp/Cp	1.967/2.566	GhOMT78	Cp	2.277
GhOMT23	Cp	4.529	GhOMT51	Cp	3.273	GhOMT79	Cp	2.154
GhOMT24	Cp	4.404	GhOMT52	Cp	4.607	GhOMT80	Cp	3.576
GhOMT25	Cp	4.069	GhOMT53	Cp	3.946	GhOMT81	Cp	2.09
GhOMT26	Cp	4.655	GhOMT54	Cp	4.695	GhOMT82	Cp	3.123
GhOMT27	Cp	3.408	GhOMT55	Pp	3.275			
GhOMT28	Cp	4.921	GhOMT56	Cp	4.391			

Cp: Cytoplasmic, Pp: Periplasmic, OM: outer membrane, IM: inner membrane

*Reliability: Lower reliability value shows the stronger possibility of predicted localization

3.7 Enrichment analysis

To understand the functional annotations of *OMT* family genes of *G. hirsutum*, 82 genes in *G. hirsutum* were undergone through gene ontology (GO) enrichment, kyoto encyclopedia of genes and genomes (KEGG Pathway), and InterPro analyses. In GO term analysis, all of the *OMT* family genes in *G. hirsutum* were enriched mainly in three molecular function categories, namely all 82 genes in O-methyltransferase activity, 62 of the 82 genes in methyltransferase activity, and 53 of the 82 genes in protein dimerization activity

(Fig. 3.a). In KEGG Pathway analysis, the *OMT* family genes are categorized into two groups according to their domain functions. Twenty-nine *OMT* family genes were involved in monolignol biosynthesis, phenylpropanoid, secondary metabolism, and metabolic pathways and eleven genes were involved in phenylalanine and flavonoid biosynthesis pathways (Fig. 3.b). In InterPro analysis (http://www.ebi.ac.uk/interpro/), 82 *OMT* genes were categorized as common as the functions of S-adenosyl-L-methionine-dependent methyltransferase (Fig. 3.c). Followed by the second most enriched categories of methyltransferase_2 and O-methyltransferase COMT-type, with Sixty-two genes predicted in each of them (Fig. 3.c). Fifty-seven *OMT* genes were enriched in winged helix-turn-helix DNA-binding domain, among which 53 genes were also predicted in plant methyltransferase dimerization category (Fig. 3.c).

3.8 Expression profiling of *OMT* genes and their homologues

In order to verify the biological functions of *OMT* family genes, several transcriptome data sets including TM-1 (Hu et al. 2019), *G. arboreum*, *G. raimondii*, CSSLs (Li et al. 2017), and RILs (Zhang et al. 2020b), were applied to analyze their expression profiles in different developmental stages, organs, or tissues, and responses to various abiotic stress treatments. The transcriptome clusters showed that the OMT genes can be assorted into three basic groups (Fig. 4A): Those that have a broad responses to different developmental stages from germination to fiber maturation, typical examples of which included *GhOMT33*, *GhOMT71*, *GhOMT16* and *GhOMT55*; those that have specific responses to root development, including *GhOMT28*, *GhOMT4* and *GhOMT34*; and those that have responses to early germination in seed, cotyledon, root and stem, including *GhOMT58*, *GhOMT32* and *GhOMT46*. When fiber specific transcriptome data sets of *G. arboreum*, *G. raimondii* were applied to observe the expression profiling diploid OMT family genes, the result also supported specific expression profiling of some OMT genes in diploid species of *G. arboreum* (Fig. 4.B) and *G. raimondii* (Fig. 4.C).

The gene expression profiling was further verified with trancriptome datasets of RILs (Fig. 5.A) two CSSLs (Fig. 5.B and 5.C). The results showed that the genes that had specific expressions during fiber development (Fig. 4.A) also had specific expressions in fiber development of RILs and CSSLs materials. These genes had a highly consistent expression profiling among the different cotton cultivars and lines during fiber development. Some selected *GhOMT* examples genes, *GhOMT16* (Fig. 5.D), *GhOMT27* (Fig. 5.E), *GhOMT33* (Fig. 5.F), *GhOMT43* (Fig. 5.G), and *GhOMT55* (Fig. 5.H), were verified through qRT-PCR using sGK9708 and 0-153, the two parental lines of the RIL population with different fiber quality traits. The results showed that *GhOMT16*, *GhOMT33*, and *GhOMT55* were significantly up-regulated during fiber development in sGK9708 than in 0-153 Fig. 5.D, 5.F and 5.H) and that GhOMT 27 and GhOMT43 did not show differences between the two cultivars (Fig. 5.E and 5.G). Noticeably, *GhOMT16* and *GhOMT55* reached the highest expression levels at 20 DPA and their high expression lasted in a short time as compared with that of *GhOMT33*. *GhOMT33* had a rapid expression increase from 10 DPA to 15DPA and then its expression steadily increased until 25 DPA when it reached its highest expression level.

Based on the expression profiling of the *OMT* gene family in responses to cold, hot, osmotic, and salt stress treatments (Fig. 6.A), two genes specific in salt stress responses, *GhOMT27* and *GhOMT43*, and three genes specific in fiber development, *GhOMT16*, *GhOMT55*, and *GhOMT33* were verified by qRT-PCR with RNA samples extracted from salt treatment. The results indicated that both *GhOMT27* and *GhOMT43* showed an elevated expression in salt treatments in salt-tolerant cultivar as compare to the control treatments (Fig. 6.B and 6.C). These two genes had different expression profiles from 2 h to 6 h after salt treatment. *GhOMT27* had the highest expression at 2 h and then its expression went down at 6 h; whereas *GhOMT43* had an increasing expression pattern from 2 h to 6 h. Both genes had much higher expression in roots than in stem or leaf.

4. Discussion

4.1 A genome-wide survey of OMTs

A genome wide search of *G. hirsutum* (Hu et al. 2019), *G. arboreum* (Du et al. 2018), and *G. raimondii* (Paterson et al. 2012) resulted in the identification of 192 genes (82 in *G. hirsutum*, 55 in *G. arboreum*, and 55 in *G. raimondii*). Recent study testified that modern allotetraploid *Gossypium* species were developed from a natural hybridization between the ancestors of two diploid species of *G. raimondii* (D-genome) (Wang et al. 2012) and *G. arboreum* (A-genome) (Du et al. 2018) 1.7 to 1.9 million years ago (Hu et al. 2019). The results of current study revealed a loss of quite a large number of *OMT* genes in *G. hirsutum* A_tD_t genome as compared to the total number of *OMT* genes in A and D genomes. Possibly 19 *OMT* genes in A_t sub-genome and 17 in D_t sub-genome in *G. hirsutum* (S4 Figure) were lost during the evolution procedure after it arised from above mentioned hybridization (Hu et al. 2019). Gene losses can be the result of premature stop codon, disruption of genes as compared to their orthologous (Gan et al. 2011), and rapid genome re-

organization during polyploidization and diploidization process (Brenchley et al. 2012; Otto 2007; Soltis and Soltis 2009). Previous studies have evidenced that polyploidization processes may result in losing of homologous members or altered expression profiles of the homologous genes or both (Cheng et al. 2012; Grover et al. 2012; Leach et al. 2014; Zhang et al. 2015). Similar phenomenon was noticed in the expression profiling of homologous *OMT* genes between A_t and D_t in *G. hirsutum*, which clued that these genes might have experienced abovementioned events during evolution processes. Collectively, a higher number of genes were also identified in dispersed duplication event. Dispersed duplication has been studied for its disruptive nature and adverse effects such as altering the gene expression (Kazazian Jr et al. 1988). Besides the dispersed duplication, segmental duplication events were also identified in a higher number of *OMT* genes. Segmental duplication is widespread in flowering plants, which might lead to the evolution of novel genes and their functions (Renny-Byfield et al. 2014).

Analyses of selection pressures and phylogenesis revealed that most of *OMT* genes in *Gossypium* species were under a purifying selection pressure and that high similarity of *OMTs* within *Gossypium* species supported the conservative evolution mode of *OMT* genes. The purifying selection pressure might suggest the importance of OMT genes in *Gossypium* species. But noticeable exceptions were also observed in some interspecific homologous pairs, in which their Ka/Ks values were above one, indicating these homologous pairs were under a positive selection pressure. These homologous pair exceptions included *GrOMT16-GhOMT78* and *GrOMT29-GhOMT25* in *G. raimondii* and *G. hirsutum*, *GaOMT21-GhOMT15* in *G. arboreum* and *G. hirsutum*, *GrOMT30-GaOMT39* and *GrOMT29-GaOMT40* in *G. raimondii* and *G. arboreum*. These results suggested that the OMT genes might had experience positive selection pressures during the evolution from diploids to tetraploids. Previous studies have evidenced that the positive selection pressure might be associated with the onsets of new functions in genes (Conant and Wolfe 2008; Van Zee et al. 2016). Considering the fact that quite a proportion of OMT genes were lost during the formation and evolution of allotetraploid cotton (see afore discussion and S4 Figure). In the current study, two *OMT* family members, *GhOMT40* and *GaOMT5*, were characterized as reticuline 7-O-methyltransferase. Since reticuline is unknown in higher plants and have been only reported in legumes (Akashi 2003; He 1998). Therefore, how these genes function is still open to discussion. Taken all findings together, the results might suggest that the *OMTs* that experienced positive selective pressure be lost or take on some novel functions in *G. hirsutum* during the processes of its evolution and ancestor formation.

Previous findings have reported that the *G. raimondii* (D-genome) and *G. arboreum* (A-genome) are the closest relatives to the D_t and A_t sub-genomes of allotetraploids, respectively (Hu et al. 2019). Each gene in A or D genome will always have a homolog in the correspondent A_t or D_t sub-genomes of *G. hirsutum* (Ge et al. 2020). However, in both A and D genomes we detected quite a large number of *OMT* genes that do not have homologs in their relative A_t and D_t sub-genomes (S4 Figure). Previous studies evidenced that such homolog loss could result from two possible reasons: one is that the homologs were lost during the procedure of polyploidization from diploids to tetraploid; the other is that after the tetraploid formation, the OMT members in each genome started their separate evolution procedure. This separate evolution procedure makes the newly evolved members have no homologs in its relative genomes (Hu et al. 2019). Previous studies revealed that in A, D, A_tD_t genomes do not maintain same speed of evolution. A faster evolution rate was observed in allotetraploid cottons than in diploid cottons (Hu et al. 2019). Taken the fact that *OMT* genes undergo a purifying selection procedures (S5 Table. Sheet B), the first reason is possibly endorsed as the main cause for the current evolution status of *OMT* gene family and the second reason may also played a role.

4.2 Function prediction of OMT candidates

4.2.1 OMTs are involved in diverse cis-regulatory elements

Plants encounter various biotic and abiotic stresses during their entire life cycles that negatively affect growth, development, and productivity (Lamaoui et al. 2018). Under exposure of these stresses, plants require some potential mechanism, which can be activated in critical circumstances, to support whole plant life cycle (Rao et al. 2006). Excessive salinity is also a major factor that affects the cotton production all around the world (Xu et al. 2013). Identification of cis-regulatory elements revealed that the *OMT* genes are enriched with important cis-regulatory elements that are essential against negative environmental stresses. Some important regulatory elements, including W-box, MYB, MYC, DRE, ABRE, G-Box, MBS (Sazegari et al. 2015), were identified in *OMT* genes. W-box is important to regulate the expression of genes and to bind *WRKY TFs*. *WRKY TFs* are important to mediate plants to defense against chilling, wounding, drought, salinity and heat stresses (Eulgem et al. 2000; Hara et al. 2000; Huang et al. 2002; Maleck et al. 2000; Meier et al. 2008; Pnueli et al. 2002; Rizhsky et al. 2004a; Rizhsky et al. 2004b; Seki et al. 2002). MYB and MYC have been identified as involved in dehydration-response (Abe et al. 1997). DRE (Thomashow 2010), which up-regulate gene expression under cold stress and increase the tolerance of plants was also identified in these specific genes. ABRE is an important regulatory element that enhances salt stress tolerance in plants. It plays a key role in dehydration and in response to salinity stress in *Arabidopsis thaliana*, soybean and rice, and in

response to chilling or cold in *Paeonia suffruticosa* (Zhang et al. 2016). G-box is identified in several gene promoters in previous studies and it contributes to development, hormone response, and tolerance against fungal infections in plants. Besides, a gibberellins response element (GARE) was also identified to be important to promote flowering in plants. The auxin hormones play a major role in growth and development of diverse plant species (Zhao 2010). These results were in accordance with our findings. Especially the repetitively identified cis-regulatory elements might have biological functions in plants under specific conditions and development stages.

4.2.2 OMTs are involved in important secondary metabolic pathways

The KEGG pathways enrichment analysis revealed the involvement of *OMT* genes in secondary metabolism and metabolic pathways including monolignol, phenylpropanoid, flavonoid, and phenylalanine metabolisms. Secondary metabolic pathways are demonstrated to have exceptional impacts on biotic and abiotic stresses. Secondary metabolites are phytochemicals, which are synthesized through secondary metabolism. In plants, phenylpropanoids are categorized in several groups such as phenolic acids, flavonoids, and lignins, which are involved in diverse physiological processes and tolerance under unfavorable conditions (Cheynier et al. 2013; Dixon 1995; Laura et al. 2009; Naikoo et al. 2019; Ramawat and Mérillon 2013). The activity of secondary metabolites increases during the response of abiotic stresses. These phenolics provide plants with higher tolerance against heavy metals (Handa et al. 2019; Smirnov et al. 2015), salinity (Weretilnyk et al. 2001), drought (Ancillotti et al. 2015), and temperature stresses (Naikoo et al. 2019). These pathways also play an important role in plant cell elongations (Fan et al. 2006; Sasayama et al. 2011). Same as, plant *OMT* genes have been identified in secondary metabolism (Wang and Pichersky 1999). Higher expression of secondary metabolic pathways related genes in developing cotton fiber is reported in previous studies (Al-Ghazi et al. 2009; Gou et al. 2007). Importantly, *OMT* genes were reported to be involved in lignin synthesis and to be induced by inoculation of *Veriticillium dahliae* in cotton (Cui et al. 2000; Grimmig et al. 1999; Ni et al. 1996). During the inoculation of pathogens, changes in the expression patterns of phenylpropanoid related *OMT* genes were identified. These identified *OMT* genes included *GhOMT49*, *GhOMT58*, *GhOMT20*, and *GhOMT61* that were found significantly expressed in 12 and 48 hours post inoculation *Verticillium dahliae* (Li et al. 2019a). In the current study, these genes were down-regulated under abiotic stresses and in fiber development stages (Fig. 4). Previous reports have evidenced that desoxyhemigossypol-6-O-methyltransferase (dHG-6-OMT) catalyzed the biosynthesis of terpenoid and provided an effective defense mechanism to cotton plant against biotic stresses including insects and pathogens (Liu et al. 1999). In response to *V. dahliae* (V991) in CSSLs lines CCRI36 and MBI8255, diverse genes were found differentially expressed in lignin biosynthesis including *CCoAOMT*, which can adequately utilize lignin and has been characterized in several previous studies (Li et al. 2019b; Wang et al. 2016). Another study also reported that *CCoAOMT* was up-regulated in response to *Verticillium* pathogen in cotton and rendered cotton plants a comparable phenotypic resistance as compared to control plants (Tang et al. 2019). A RNA-seq analysis based research identified differential expression patterns of *CCoAOMT* in response to *V. dahliae*, confirming the effect of this *OMT* gene in the plant response to *V. dahliae* in cotton (Xu et al. 2011). These results consequently evidenced the important role of secondary metabolic pathways and *OMT* genes in biotic stresses in cotton.

4.2.3 OMTs are involved in plant growth, abiotic stress tolerance, and fiber development of cotton

Salinity is one of the major causes to reduce crop yield (Tuteja 2007) and incurs up-regulation and/or down-regulation of plant genes in response (Joshi et al. 2009). The *OMT* genes have been found specific for salt stress tolerance and fruit development in tomato plant (*Solanum lycopersicum*) (Liu et al. 2019). The SAM-dependent methyltransferases genes were identified to play important role in sweet potato (*Ipomoea batatas*) in response to salt stress (Liu et al. 2015). In wheat, *TaCOMT-3D* contributes to stem mechanical support (Wang et al. 2018). Another *TaCOMT* gene was also observed with constitutive expression in stem along with leaf and root (Ma 2009). The *OMT* gene (*BdCOMT1*) was strongly expressed in stem node and internode but poorly expressed in other tissues in *Brachypodium distachyon* plant (Wu et al. 2013). The expression profiles of *OMT* gene family in the transcriptome data of TM-1 (Hu et al. 2019) and verification results through qRT-PCR also suggested that two *OMT* members *GhOMT43* and *GhOMT27* might contribute to salt stress tolerance in *G. hirsutum*. In the qRT-PCR verifications, *GhOMT43* and *GhOMT27* showed different expression profiling from 2 h and 6 h after 200 mM NaCl treatment (Fig. 5). Probably they act differently in response to salt stress in *G. hirsutum*. Four genes including *GhOMT28*, *GhOMT38*, *GhOMT32*, *GhOMT62*, and *GhOMT26* had significant expressions in stem (Fig. 4.A) where they might be the potential candidates to provide structural support and survival to plant in environmental stresses.

Cotton fiber quality of is an important attribute to develop elite cultivars in the presence of negative environmental factors. Studies demonstrated that *GhOMT16* and *GhOMT33* were expressed at elongation stages of a CSSL (CS-B25) and TM-1 respectively (Hsu et al. 2018; Hu et al. 2019). In the current study, the fiber specific *OMT* genes were consistently identified across various populations and species including TM-1 (Fig. 4.A) *G. arboreum* (Fig. 4.B) *G. raimondii* (Fig. 4.C), RILs (Fig. 5.A), CSSLs (Fig. 5.B, 5.C), and. They also showed highly similar expression patterns in different fiber development stages. The expression specificities of *GhOMT16*, *GhOMT33*,

and *GhOMT55* in developing fibers were further verified through qRT-PCR studies (Fig. 5.D, 5.F and 5.H). The results demonstrated that these *OMT* members could have a significant function in fiber development and fiber quality formation. But how these genes function during fiber quality formation was still open to discussion.

Lignins-like phenolics are widely studied in response to stress (Bhardwaj et al. 2014). Recent research advancements revealed that lignin or phenolics influence fiber development at elongation and secondary cell wall synthesis stages (Han et al. 2013). The knock-down of Lignin-like phenolics related gene (*GhbHLH18*) in *G. hirsutum* evidenced the regulation of lignin-like phenolics pathway genes including a *COMT* and others, during cotton fiber elongation and secondary cell wall synthesis stages. The results demonstrated the roles of these genes in regulating the lignification in developing cotton fibers (Gao et al. 2019). This study has gathered important information of *OMT* gene family which is a forward step in research to uncover the possible functions or to support previous studies in exploration the functions of *OMT* genes in plant response to salt stress and in cotton fiber development.

5. Conclusions

Methyltransferases are versatile class of enzymes. *OMT* contributes to diverse phenolics that are essential for plant growth and serves as protective shield against several kinds of stresses. Various bioinformatics analyses revealed that *OMT* gene family is a strong growth regulator, which not only provide protection to the plant, but also are involved in fiber elongation and secondary cell wall synthesis stages. Furthermore, expression profiling analysis based on several transcriptome data and qRT-PCR validation inferred that *GhOMT43* and *GhOMT27* might be the potential candidates for salt stress tolerance and that *GhOMT16*, *GhOMT55*, and *GhOMT33* might have significant influence in fiber development at elongation and secondary cell wall stages of *G. hirsutum*. This proposed study concludes the important roles of *OMT* family genes in cotton fiber development and in salt stress tolerance.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Competing interests

Authors declare that they have no competing interests for the publication of the manuscript.

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Figures

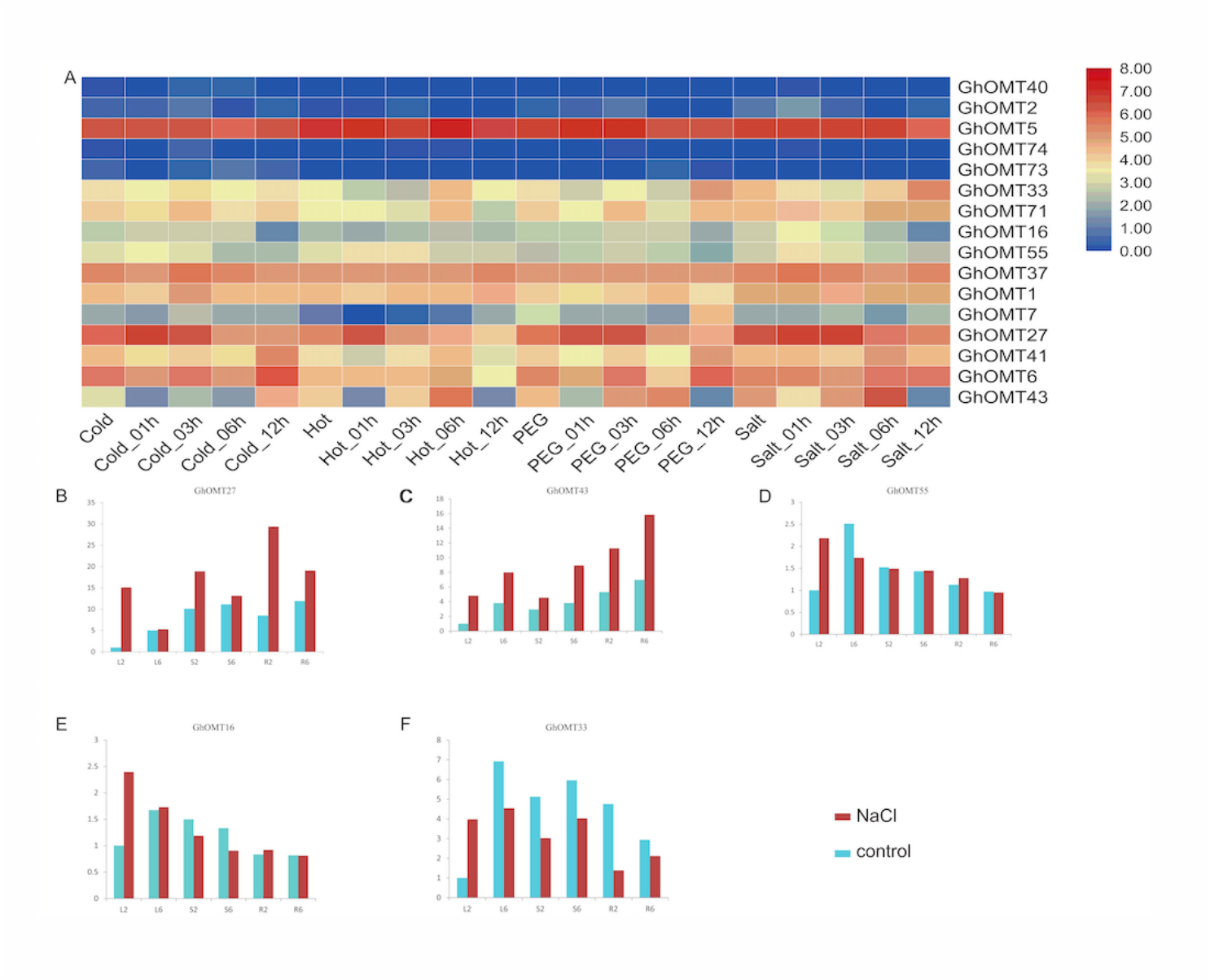

Figure 1

Specific responses of selected OMTs in salt stress treatment. A: Transcriptome heatmap of selected GhOMT genes in cold, hot, osmotic and salt treatments (Hu et al. 2019; Zhang et al. 2015). B-F: qRT-PCR verification results of GhOMT27, GhOMT43, GhOMT55, GhOMT16, and GhOMT33 in salt treatments of sGK9708.

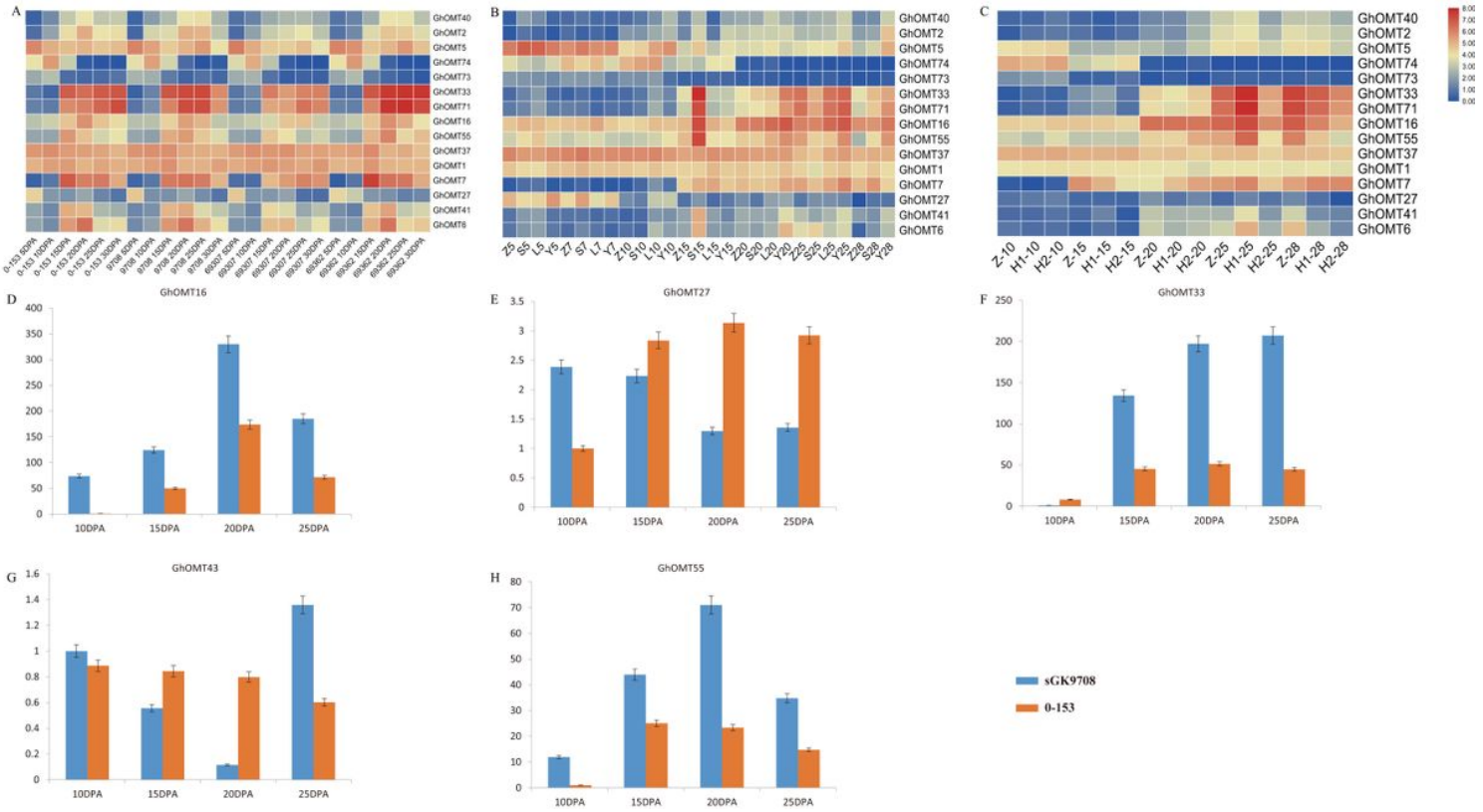

Figure 2

Specific responses of selected OMT genes in fiber development. A: Transcriptome heatmap of selected GhOMT genes in RIL lines and their parents (Zhang et al. 2020b). B: Transcriptome heatmap of selected GhOMT genes in CSSLs of CCRI45 and Hai1 (Lu et al. 2017; Shi et al. 2015); Z, S, L, and Y represent CCRI45, MBI7561, MBI7747, and MBI7285, respectively; 5, 7, 10, 15, 20, 25, and 28 represent different DPA. C: Transcriptome heatmap of selected GhOMT genes in CSSLs of CCRI36 and Hai1 (Li et al. 2017; Shi et al. 2015); Z, H1, and H2 represent CCRI36, MBI9915, and MBI9749; 10, 15, 20, 25, and 28 represent different DPA. D-H: qRT-PCR verification results of GhOMT16, GhOMT27, GhOMT33, GhOMT43, GhOMT55 in developing fibers of sGK9708 and 0-153 at 10, 15, 20, and 25 DPAs.

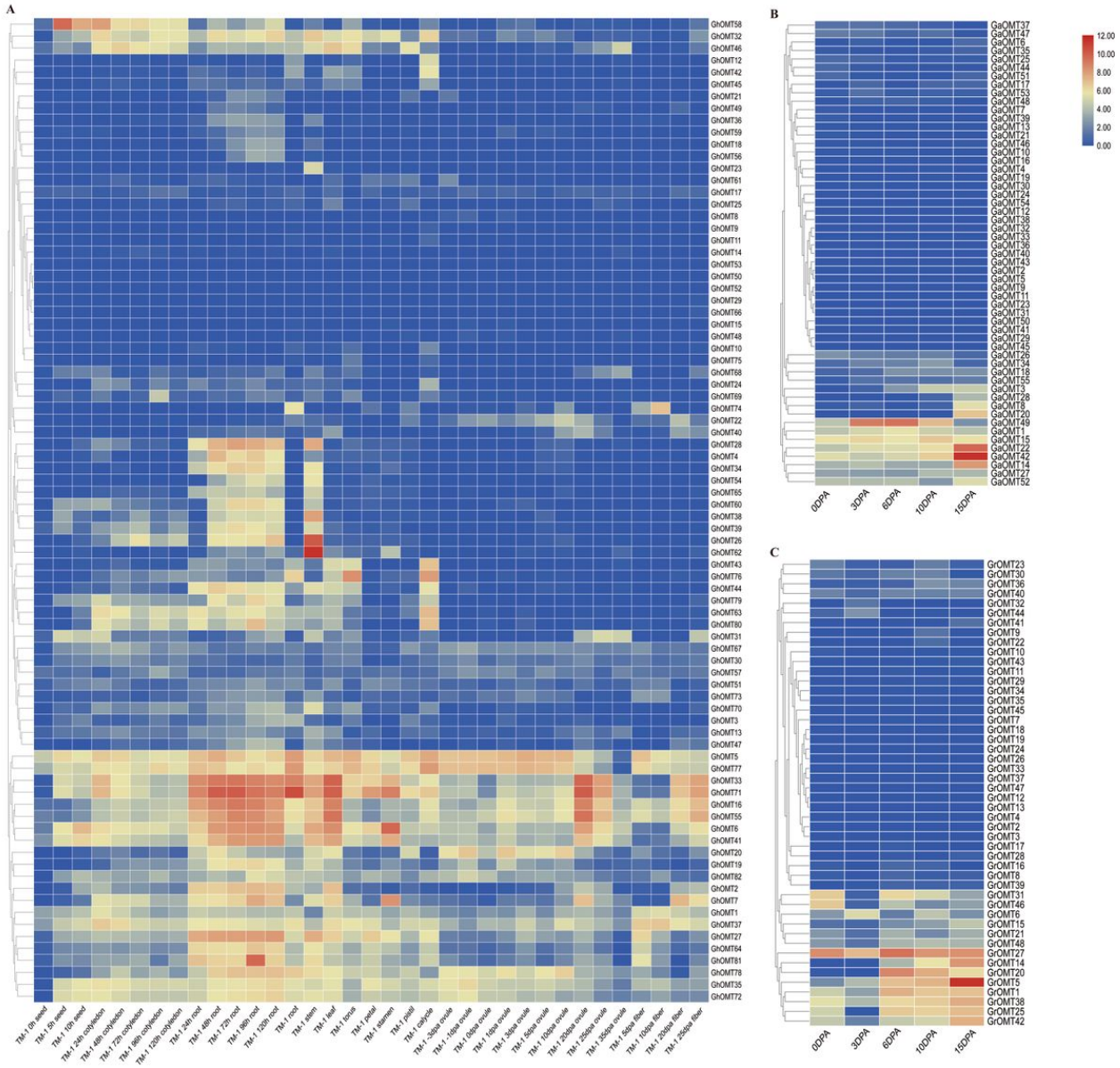

Figure 3

Transcriptome analysis of OMT family genes in different growth, ovule and fiber development stages. A: Transcriptome heatmap of GhOMT family genes in standard genetic cultivar TM-1 at different growth, ovule and fiber development stages (Hu et al. 2019; Zhang et al. 2015). B: Transcriptome heatmap of GaOMT family genes in G. arboreum (Zhang et al. 2015). C: Transcriptome heatmap of GrOMT family genes in G. raimondii (Wang et al. 2012).

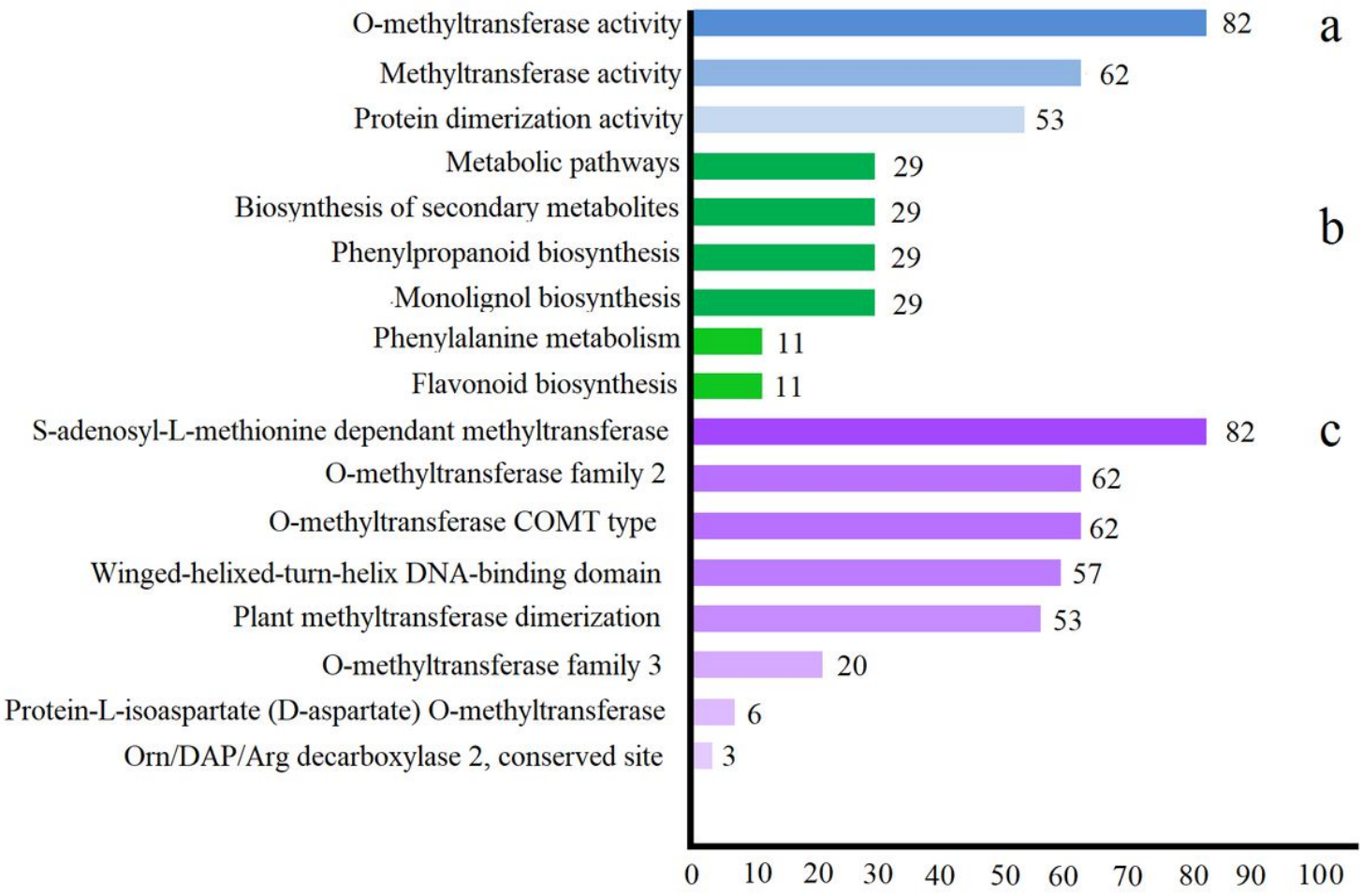

Figure 4

Enrichment analysis of OMT family genes in G. hirsutum. a. The functional annotations of Gene Ontology. b. The functional annotations of KEGG pathways. c. The functional annotations of Interpro. The scales indicate the enriched gene number in respective categories.

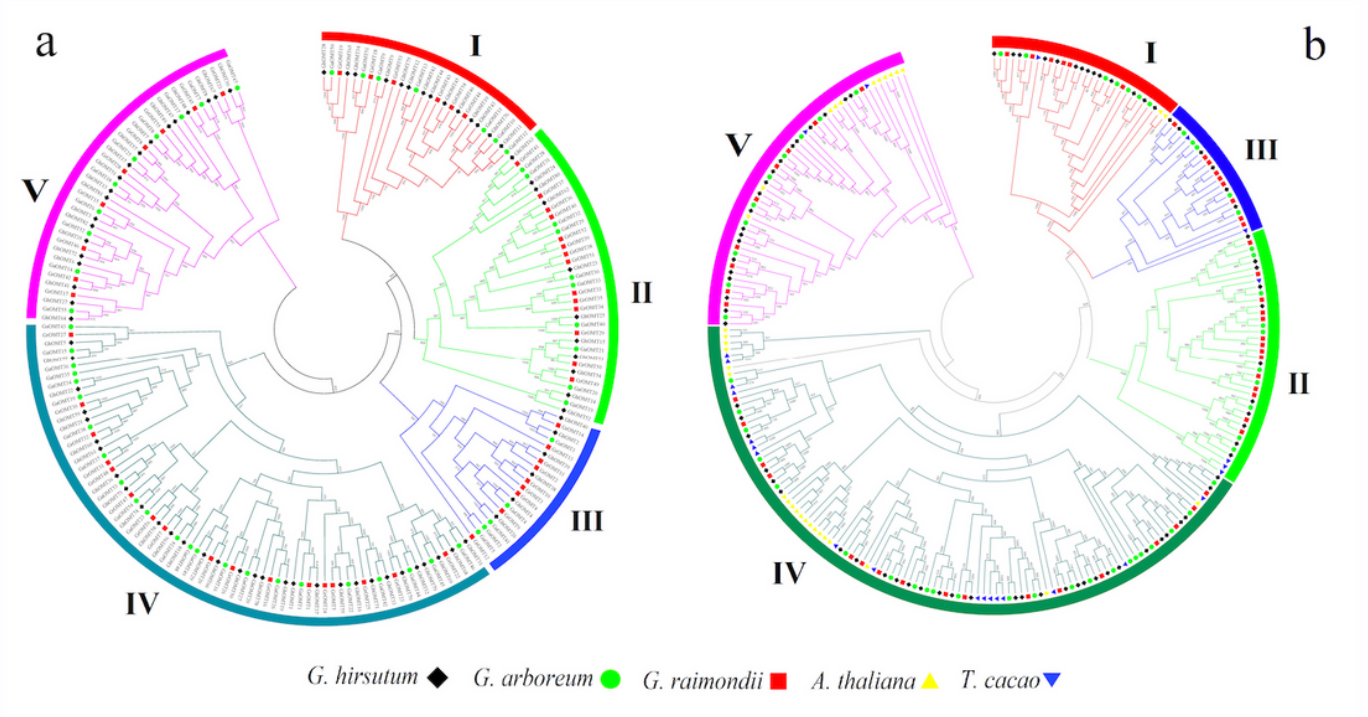

Figure 5

Phylogenetic analysis of OMT genes in Gossypium, A. thaliana, and T. cacao species. (a) Neighbor-joining phylogenetic tree of 192 OMT genes of G. hirsutum, G. arboreum, and G. raimondii. (b) Neighbor-joining phylogenetic tree of 251 OMT sequences of G. hirsutum, G. arboreum, G. raimondii, A. thaliana, and T. cacao. I, II, III, IV and V indicate the five groups of phylogenetic tree. Shapes with different colors represent OMT members of G. hirsutum, G. arboreum, G. raimondii, A. thaliana, and T. cacao.

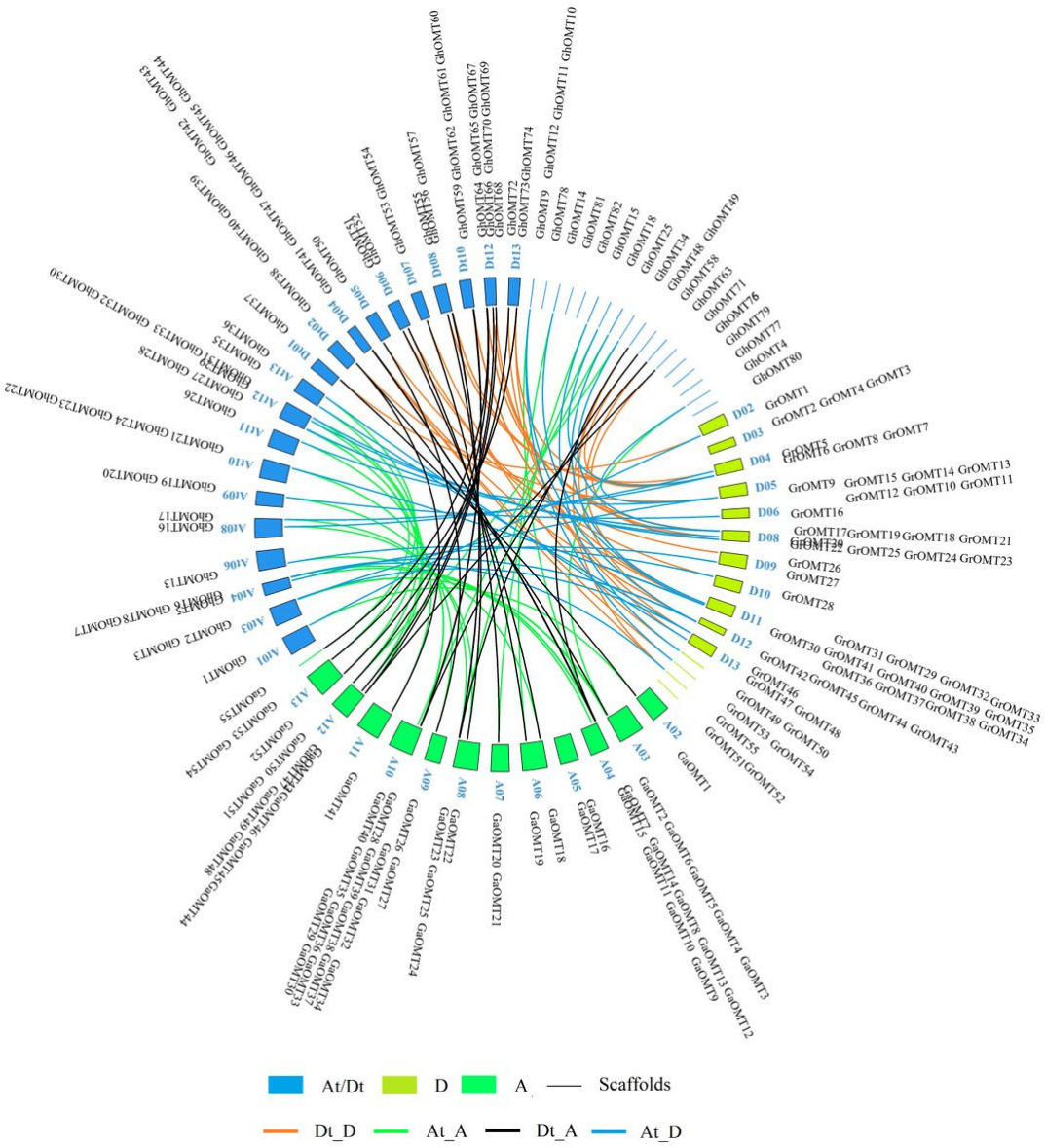

Figure 6

Collinearity analysis of OMT genes between AtDt (G. hirsutum), A (G. arboreum), and D (G. raimondii) genomes.

Supplementary Files

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